REMARKS

The amendments to the specification are being made to add the Sequence ID. Nos. For the Examiner's convenience, a copy of pages 3, 4, 15, 16 and 18 of the orginally filed specification are attached.

Respectfully submitted,

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Brief Description of accompanying drawings :

1. Photographs 1 and 2 of drawing sheet 1 show field view of morphological features of the plants of the present invention at 70 days and 100 days respectively.
2. Photographs 1 and 2 of drawing sheet No. 2 show the rapid growth of the 'Sambhav' plant and its canopy at 70 days and 100 days respectively.
3. Photograph 1 of drawing sheet No.3 shows instar larvae infected individual clones of other plants in comparison with clone of 'Sambhav'.
4. Photograph 2 of drawing sheet No.3 shows the unique RAPD profile of 'Sambhav'.

Detailed description:

Breeding history :

Bihar hairy caterpillar (*Spilarctia obliqua*) is a major pest of polyphagus nature which infects heavily over a large area of mint crops frequently in Terai and north Indian plains. The infestation sometimes is so heavy and unmanageable that it may lead to more than 80% to complete loss of foliage and consequently the oil yield proportionately. Hence it is desirable to explore the possibility of developing insect tolerant high yielding clones in mints. The popular menthol mint variety "Himalaya" developed in 1996 by CIMAP (US Pat. No PP10935) was, therefore, used as the starting material for specific improvement towards insect tolerance through generation of somaclonal variation and simultaneously looking for improved plant type with higher essential oil and menthol yields.

Encouraged by the initial leads in the laboratory for rapid detection and isolation of somaclonal variants by using the protocol reported by us (S. P. S. Khanuja, A. K. Shasany, S. Dhawan, S. Kumar, Rapid procedure for isolating somaclones of altered genotypes in *Mentha arvensis*. J Med. Aroma. Plant Sci. 20 (1998) 359-361), we generated 3000 independent somaclones. These clones were subjected to detection of molecular variation at the tissue culture stage itself through RAPD profiling. DNA

was isolated from 40 mg of leaf tissue and Polymerase chain reactions (PCRs) were carried out in 25 µl volume. A reaction tube contained 25 ng of DNA, 0.2 unit of Taq DNA polymerase , 100 µl each of dNTPs , 1.5 mM $MgCl_2$ and 5 p mol of decanucleotide primers. The amplifications were carried out using a thermal cycler (MJ Research, USA). The amplified products were loaded in 1.2% agarose gel containing 0.5 µg ml^{-1} of ethidium bromide and photographed by Polaroid system. Twelve decamer primers having the sequences AAATCGGAGC, GTCCTACTCG, GTCCTTAGCG, TGCGCGATCG, AACGTACGCG, GCACGCCGGA, CACCCTGCGC, CTATCGCCGC, CGGGATCCGC, GCGAATTCCG, CCCTGCAGGC, CCAAGCTTGC were used to analyse all the *in vitro* regenerated clones. Out of 3000 regenerated clones 245 showed variation at DNA level in the RAPD profiles compared to the control plant "Himalaya".

The individual molecular variants selected through RAPD analysis of somaclones as above were then subjected to screening against the larvae of lepidopteran insect pest *Spilarctia obliqua* .

For this purpose, a new strategy was devised by subjecting the *in vitro* growing clones to attack by actively feeding 3rd instar larvae by releasing them right in the culture tubes containing individual clones on the rooting medium (Sheet # 3, Photograph # 1). Most of the shoots of the clones were eaten away by these larvae within 2-3 days. However, three clones showed the least feeding by the larvae. In these tubes, only initial bites could be observed and nonfeeding was also conspicuous by typical symptoms of stalled growth in the starved larvae. These larvae were then transferred to other clone tubes, where they resumed feeding. This led to the applicants to believe that the three clones must have some characteristics not liked by the feeding larvae. So the applicants again confirmed this by releasing another set of actively feeding 4th instar larvae into the tubes containing these three identified clones. This process was repeated three times and each time, the larvae showed non-preference and stopped feeding.

The applicants then hardened these three "insect-non-preferred" clones namely, CIMAP/GRB 1-06, 2-18 and 5-15 and transferred to the glasshouse in pots. Among these three clones, CIMAP/GRB 2-18 showed conspicuously vigorous growth

The plant genotype "Sambhav" developed in the present invention is a herbaceous perennial with a single tall upright stem possessing several lateral branches coming out from the lower nodes laterally rising in a fashion to give a shape of an open filled umbrella turned upside down.. This special arrangement of branches facilitates the distribution of the captured sunlight equally to all the leaves and hence avoiding shading thereby, reducing lower leaf fall amounting to the prevention of economic loss to the plant. The chromosome number of the plant is 2n=96. The colour codes are in accordance with the "RHS colour chart published by the Royal Horticultural Society, 80 Vincent Square, London SW1P 2PE,1995.

Evidence of uniformity and stability

No variants of any kind (morphological or molecular) has been observed since 1997 indicating the stability and uniformity of the genotype. Further, the comparative herbage and oil yields of "Sambhav" were significantly higher in comparison to other varieties/genotypes in different years and seasons. Due to vigorous vegetative growth this genotype can be harvested earlier without reducing the yield of herbage, oil or menthol. The traits of insect tolerance against *S. obliqua* is unprecedented and stable.

Statement of distinction

The genotype "Sambhav" possessing a very high level of insect tolerant character against leaf damage by *S. obliqua* larvae is unique and unprecedented not possessed by any known variety. Additionally, it has a distinct canopy of one straight main stem with many lower branches arranged like an open filled umbrella turned upside down which is characteristic to this genotype only. The genotype is having highest biomass and highest oil yield unit area in comparison to others. The total menthol yield of the new genotype is higher per unit area in comparison to other genotypes. Its genetic make up is distinct in terms of DNA profile.

Randomly Amplified Polymorphic DNA analysis : The RAPD profiles of the plant "Sambhav" were unambiguously able to establish its distinct identity as completely different from the parent plant "Himalaya" as well as the known released

varieties. The plant of the present invention was developed by screening molecular variants among somaclones already differentiated as distinct, unique and novel at DNA level. The plant is having desirable morphological and economical traits in a rare unmatchable combination and is available only with us in CIMAP. No variation in the RAPD patterns was observed in the analysis of the micropropagated as well as field raised population in successive generations indicating the stability of the genotype. The 20 MAP primers (MAP 01 to MAP 20) with the sequence AAATCGGAGC, GTCCTACTCG, GTCCTTAGCG, TGCGCGATCG, AACGTACGCG, GCACGCCGGA, CACCCTGCGC, CTATCGCCGC, CGGGATCCGC, GCGAATTCCG, CCCTGCAGGC, CCAAGCTTGC, GTGCAATGAG, AGGATACGTG, AAGATAGCGG, GGATCTGAAC, TTGTCTCAGG, CATCCCGAAC, GGACTCCACG, AGCCTGACGC and 20 OPJ primers (Operon Technologies Inc, USA) were used for the analysis and similarity indices were computed to generate similarity matrix among existing varieties and the plant Sambhav (Table 3). The OPJ primers (01 to 20) were procured from Operon technologies, USA. The MAP primers were used to develop a unique and distinct RAPD profile (Drawing sheet # 3, Photograph # 2)of the Plant.

Table 3. Similarity indices of different control plants analysed in comparison to Sambhav

Gomti	Himalaya	Kosi	MAS-1	Kalka	Shivalik	Sambhav
1.00						
0.90	1.00					
0.89	0.94	1.00				
0.91	0.92	0.92	1.00			
0.85	0.88	0.85	0.89	1.00		
0.92	0.93	0.91	0.90	0.87	1.00	
0.87	0.73	0.78	0.82	0.83	0.84	1.00

So the protocol for developing insect tolerant plants *in vitro* can be explained in details as an example of the development of 'Sambhav' which involves following steps and sub-steps.

Example of development of insect tolerant plant 'Sambhav'.

Step A: Explant, culture conditions and regeneration for generating genetically modified somaclones.

1. The *Mentha arvensis* cv Himalaya explant material was collected from the field grown plants and washed sequentially with 2% detergent, distilled water containing a few drops of Savlon (Johnson and Johnson, India), 0.1% acidified mercuric chloride and autoclaved distilled water. About 1cm long internode pieces were inoculated in the MS media (Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Planta.* **15**:473-497.) containing the auxin, 0.2μg ml^{-1} 2,4 dichlorophenoxy acetic acid (2, 4-D) and the cytokinin, 7μg ml^{-1} 6-(γγ'- dimethylallyl amino) purine (2iP or 2aP).
2. The cultures were grown at 25±2°C and 400 to 600 lux light intensity with 16 h photoperiod.
3. The regenerated shoots were separated at 12 weeks from the explant inoculation and transferred to the MS medium free of hormones for rooting.
4. The plantlets thus generated were examined for any genotypic change by comparing their RAPD profiles with that of cv Himalaya using the 12 random decanucleotide primers having the sequences AAATCGGAGC, GTCCTACTCG, GTCCTTAGCG, TGCGCGATCG, AACGTACGCG, GCACGCCGGA, CACCCTGCGC, CTATCGCCGC, CGGGATCCGC, GCGAATTCCG, CCCTGCAGGC, CCAAGCTTGC.
5. We obtained 245 molecular variants after screening 3000 regenerated clones. Out of 245 clones 155 clones were different from 'Himalaya' with one primer out of the 12 primer used. Eighty-two variants were different from 'Himalaya' with two primers and the rest 8 variants with three or more primers.